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<p>(54) Title: METHOD OF PREPARATION AND USE FOR ZONA PELLUCIDA ANTIGENS AND ANTIBODIES FOR STERILIZATION AND CONTRACEPTION</p>					
<p>(57) Abstract</p> <p>The invention relates to antigenic preparations useful for inducing the production of antibodies in an individual which will bind to epitopes on zona pellucida. Also disclosed are immunogenic compositions and methods for immunizing an individual to enable the production of antibodies to zona pellucida antigens. Also disclosed are the use of these recombinant molecules and monoclonal antibodies thereto for immunocontraception or sterilization.</p>					

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METHOD OF PREPARATION AND USE
FOR ZONA PELLUCIDA ANTIGENS AND ANTIBODIES FOR
STERILIZATION AND CONTRACEPTION

BACKGROUND OF THE INVENTION

Field of the Invention

20 The present invention relates to the preparation and use of zona pellucida (ZP) antigens and monoclonal antibodies for contraception or sterilization in individuals. The present invention also relates to immunological contraception. More specifically, the 25 present invention relates to immunological contraception utilizing zona pellucida antigens produced by recombinant DNA technology to actively immunize an individual against pregnancy, or monoclonal antibodies prepared against the zona pellucida antigens to passively immunize the individual. In addition, the present invention relates to 30 the use of anti-idiotypic monoclonal antibodies which would mimic ZP antigens to actively immunize an individual against pregnancy.

35 This invention also relates to monoclonal anti-zona pellucida antibody, to novel hybridoma cells

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1 which express such antibody, and to a method for producing
such hybrid cells and anti-zona pellucida antibody.

Brief Description of the Background Art

5 The zona pellucida is the complex extracellular
glycoprotein matrix which surrounds the mammalian oocyte.
This matrix is formed during the early stages of oocyte
growth and follicular cell differentiation and serves to
protect the oocyte and embryo until implantation in the
10 uterine wall (Austin, et al., Reproduction in Mammals:
Germ Cells and Fertilization, Cambridge Univ. Press,
Cambridge, England (1982)). In addition, the zona
pellucida plays an important role in the fertilization
process since the sperm must first adhere to and penetrate
15 the zona pellucida. After binding to the zona pellucida
of an oocyte, the sperm must penetrate the zona
pellucida. Sperm penetration of the zona pellucida is
probably mediated by the limited hydrolysis of zona
pellucida components by sperm enzymes such as acrosin
20 (McRorie et al., Ann. Rev. Biochem. 43: 777 (1974); Dunbar
et al., Biol. Reprod. 32:619 (1985), and Stambaugh, Gam.
Res. 1:65, 1978). The zona pellucida remains intact after
fertilization, to ensure proper embryonic development and
perhaps to prevent embryo fusion in the oviduct (Mintz,
25 Science 138:594, 1962).

Finally, the zona pellucida plays a role in the
block to polyspermy. In some mammalian species,
fertilization alters sperm binding to the zona and its
resistance to proteolytic digestion (Austin and Braden, J.
Exp. Biol. 33:358, 1956).

30 There are major biological, morphological,
physiological and immunochemical variations in properties
among the zona pellucida of rodents as compared with the
zona pellucida of other species including sub-human
primates and humans. See reviews by Dunbar, In Mechanism
35

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1 and Control of Fertilization (J. Hartmann, ed.), Academic
Press, New York, pp. 139 (1983); Dunbar, In International
Congress on Reproductive Immunology (Wegmann and Gill III,
eds.), Oxford Univ. Press, London, pp. 505 (1983) and
5 Dunbar et al., In: Modern Cell Biology 3, (Satir, ed.)
Alan R. Liss, New York, pp. 77 (1984). Although mammalian
zona pellucida is composed of a limited number of major
glycoproteins (Bleil et al., Dev. Biol. 76:185 (1980);
Dunbar et al., Biol. Reprod. 24:1111 (1981); Timmons and
10 Dunbar, Biol. Reprod. (1987)), the structural and
functional relationships of zona pellucida proteins of
different species vary.

Attempts to develop an effective and economical
method of immunocontraception have been hindered by the
15 lack of sufficient material to produce quantities of
antigen or antibodies needed to produce a vaccine which
would either inhibit fertilization of the oocyte by the
sperm, prevent implantation of a fertilized egg, or
prevent the development of the ovaries thereby making the
20 animal permanently sterile. Early attempts to develop
immunocontraceptive methods have not been very
successful. These attempts have included the use of
naturally occurring circulating peptide hormones such as
human chorionic gonadotrophin (hCG) and follicle
25 stimulating hormone (Griffin, In Immunological Approaches
to Contraception and Promotion of Fertility, G. P. Taiwan,
Ed., Plenum Press, New York (1986)). Immunocontraception
utilizing antibodies against normally "circulating"
antigens poses the problem that immune complexes might
30 form which would bring about undesirable tissue damage.
Furthermore, immunization with "circulating" antigens has
not proven totally effective in inhibiting fertility.

35 Immunologically based methods of contraception
are preferable to other commercially available methods
such as surgical sterilization or birth control pills (for

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1 humans and pets) in which there is a continuous expense
for medication which must be used and purchased on a
regular basis and are only indicated for use on a
temporary basis. Thus, a considerable need exists for
5 antigen preparations that can induce transient or
permanent contraception in an individual and which can be
provided in a safe, reliable and cost effective manner.

SUMMARY OF THE INVENTION

10 The work of the inventor has focused on the use
of purified zona pellucida antigens for immunological
sterilization or contraception. Immunization with zona
pellucida antigens has distinct advantages over other
immunological contraceptive methods. Immunocontraception
15 with zona pellucida antigens can be designed so that it is
not abortive but instead inhibits fertilization. This is
particularly desirable for immunocontraception in humans.
In addition, this method may be modified so that ovarian
follicular development is inhibited causing permanent
20 sterilization. This modification will allow non-surgical
sterilization of pets and other animals without the
concurrent surgical risks and expense.

Another distinct advantage to zona pellucida
protein immunocontraception resides is the fact that low
25 liters of zona pellucida antibodies will block
fertilization. This is due to the localized and specific
nature of the site of action of the immunocontraceptive
antibody and the limited occurrence of naturally occurring
zona pellucida protein in the individual in which
30 fertilization is to be blocked. The zona antigens studied
to date are tissue specific and are fixed in the ovary so
they do not circulate. In contrast, the hormone proteins
circulate throughout the individual, occur at much higher
levels, and the levels of circulating antigen vary greatly
35 depending upon the physiological state of the individual.

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1 In addition, the levels of sperm antigens which must be
blocked for sperm antigen immunocontraception to be
efficient is variable, being dependent upon the amount of
sperm in the vaginal canal and uterine cavity.

5 Since the ZP proteins of a variety of animal
species are immunologically crossreactive, the necessity
for developing an immunizing antigen for each species in
which contraception is desired is obviated.

10 One object of the present invention was the
development of an effective immunological method of
contraception which (1) requires only one (or minimal)
numbers of administrations and therefore does not require
the continuous need for a physician (if human use) or
veterinarian (if animal use) and (2) can be designed to
15 induce permanent sterilization or castration (desirable in
pets) or transient infertility (desirable in humans as
well as breeding pets).

20 One of the disadvantages prior to the present
invention was the limited supply of zona pellucida
antigens and antibodies to use for immunocontraception.
25 The present invention provides a plentiful readily
available source of zona pellucida antigens and antibodies
for immunocontraception by providing zona pellucida
antigens produced by recombinant DNA technology and
monoclonal zona pellucida antibodies and ZP anti-idiotypic
antibodies. This provides an additional cost effective
advantage since antigens and antibodies are able to be
manufactured on a large cost effective scale using
recombinant DNA and hybridoma technology.

30 The present invention relates to antigenic
preparations and methods of immunizing an animal to induce
antibodies which react with epitopic determinants found on
zona pellucida antigens. This invention also relates to
monoclonal anti-zona pellucida antibody, to novel
35 hybridoma cells which express such antibody, and to a

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1 method for producing such hybrid cells and anti-zona
pellucida antibody. In addition, the present invention
relates to anti-idiotype zona pellucida monoclonal
antibodies and the use thereof for active
5 immunocontraception.

It is established that an individual antigen
(e.g., a glycoprotein) may have multiple antigenic
determinants or "epitopes" which can be recognized by
antibodies. These epitopes may include amino acid
10 sequences, carbohydrate residues, conformational or
"shape" determinants, or the site at which two different
molecules or peptides interact. The glycoproteins of zona
pellucida structure contain all of these types of
determinants (Drell and Dunbar, Biol. Repro. 30:445
15 (1984); and Timmons et al., Biol. Repro. 36:1275 (1987)).

In a primary embodiment of the invention, an
antigen preparation is produced which contains the
polypeptide portion of the zona pellucida protein antigens
using recombinant DNA techniques. In another embodiment
20 of the invention, an antigen preparation which contains
the polypeptide determinant site of zona pellucida protein
is produced using recombinant DNA techniques. These
recombinant polypeptides, and analogs thereof, are
hereinafter referred to collectively as recombinant zona
pellucida protein(s).

25 The term "zona pellucida" protein is intended to
include polypeptides having the same amino acid sequence
as the naturally occurring and recombinant zona pellucida
protein(s) and analogs thereof. The term "analog" is
intended to include proteins or polypeptides which differ
30 from zona pellucida protein by addition, deletion or
substitution of one or more amino acids providing that
said polypeptide demonstrates substantially the antigenic
and biologic activity of zona pellucida protein. These
analog include selected determinant sites of the zona

1 pellucida protein. These antigenic preparations can be
used to immunize an animal such that antibodies are
produced thereto.

5 Pharmaceutical compositions comprising the
antigen preparation of the invention and immune
response-enhancing components, together with
pharmacologically appropriate carriers, are also included
in this invention. Thus, in one embodiment the invention
comprises a substantially purified polypeptide comprising
10 the amino acid sequence of the zona pellucida protein or
parts thereof, expression vehicles comprising a DNA
sequence coding for said zona pellucida protein, hosts
transformed with said expression vehicle, methods of
producing the zona pellucida protein in hosts, and methods
15 of inducing the production of antibodies in an animal to
zona pellucida antigens comprising immunizing said animal
with a pharmaceutical composition comprising the
recombinant zona pellucida protein.

20 In another embodiment, the present invention
comprises monoclonal anti-zona pellucida antibodies, novel
hybridoma cells which express such antibodies and to a
method of immunocontraception utilizing such monoclonal
antibodies.

25 Fusion between myeloma cells and spleen cells
from immunized donors has been shown to be a successful
method of deriving homogeneous antibodies. Thus,
continuous cell lines of genetically stable hybridoma
cells capable of producing large amounts of monoclonal
30 antibodies against malignant tumors and specific viruses
and their antigenic determinants have been developed.
According to U.S. Patent No, 4,172,124 to Koprowski et
al., antibodies demonstrating a specificity for malignant
tumors can be produced by somatic cell hybrids between
hypoxanthine phosphoribosyltransferase deficient myeloma
35 cells and spleen or lymph cells derived from an animal

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1 previously primed with tumor cells. Also, according to
U.S. Pat. No. 4,196,265 to Koprowski et al., continuous
cell lines of genetically stable fused cell hybrids
capable of producing large amounts of monoclonal
5 antibodies against specific viruses and their antigenic
determinants have been developed.

Such cell fusion techniques can also be employed
to provide a reliable and standard supply of anti-zona
pellucida antibodies, e.g., immunocontraceptive
10 antibodies.

15 Immunization of an individual may be achieved
actively or passively. The term "active immunization"
means that an antigen or immunogen is administered to an
individual and the individual's immune system produces
antibodies against the antigen. The immunizing antigen
may be any substances to which a body will produce
20 antibodies. Antigens effective in the present invention
to actively immunize an individual to produce
contraception or sterilization include recombinantly
produced ZP antigen (rZP), whether glycosylated or not, as
well as anti-idiotypic ZP antibodies. The term "passive
25 immunization" means that antibodies produced outside of
the individual in vitro or in another individual are
administered to the individual in order to produce
immunocontraception. The ZP monoclonal antibodies of the
present invention when administered to an individual
30 produce such passive immunization.

35 Passive immunization with monoclonal antibodies
to ZP antigens causes transient (preferably several
months) infertility. The anti-zp monoclonal antibody
inhibits fertilization by interfering with sperm binding
to or penetration of the zona pellucida without having
adverse effects on ovarian function. Thus, the method of
immunocontraception of the present invention may be
effected passively by administration of monoclonal

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1 antibodies directed agent zona pellucida antigens or
1 actively by administration of zona pellucida antigens
 produced by recombinant DNA technology or anti-idiotype
 monoclonal antibodies.

5 When a homogeneous antibody (e.g., a monoclonal
 antibody) is used as an antigen, portions of the molecule
 may be recognized as antigenic determinants by the
 responding immunized host. The unique combination sites
 of the homogeneous antibody which would recognize its
10 antigenic determinants is termed the "idiotype."
10 Antibodies produced against these sites of the antibody
 are therefore termed "anti-idiotype." These antibodies
 may have "internal images" and therefore can have
 activities which mimic the original immunogen (Sege, K.
15 and Peterson, P.A., Proc. Soc. Natl. Acad. Sci. (USA)
15 75:2443 (1978); Schreiber et al., Proc. Soc. Natl. Acad.
 Sci. (USA) 77:7385 (1980)).

20 A monoclonal antibody (PSI) which recognizes a
 carbohydrate moiety of ZP which will inhibit sperm from
 binding to the surface of the zona pellucida as shown in
20 Figure 8. The PSI monoclonal antibody may be used as an
 immunogen (antigen) for the production of anti-idiotype
 antibodies which can then be used for immunological
 sterilization or contraception.

25 The anti-zona pellucida monoclonal antibody
 binding determinant genes may be cloned and modified by
 recombinant DNA technology to produce "single chain
 antibodies" directed against ZP antigenic determinants
 (Cabilly et al., Proc. Acad. Sci. USA 81:3273 (1984); Boss
30 et al., Nucleic Acids Research 12: 3791 (1984)).

BRIEF DESCRIPTION OF THE FIGURES

35 Figure 1 demonstrates the identification of
 antibodies recognizing ZP antigens used to screen the
 library by 2D-PAGE gel immunoblot method.

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1 Figure 2 shows the N-terminal amino acid sequence
of 3 pig ZP proteins and 2 rabbit ZP proteins.

5 Figure 3 restriction endonuclease cleavage map of
the λ gt11 vector which is used to develop the expression
library to clone the cDNA for ZP.

10 Figure 4 shows the DNA sequence of 3 clones which
express ZP antigen.

15 Figure 5 shows a northern blot analysis
demonstrating presence of ZP RNA in ovary but not other
tissues.

20 Figure 6 demonstrates SDS-PAGE Immunoblot of
 λ gt11DNA from clone P1.

25 Figure 7 shows results of active immunization on
ovarian function in rabbits.

30 Figure 8 demonstrates that monoclonal antibody
PSI inhibits sperm binding to the ZP.

BRIEF DESCRIPTION OF THE PREFERRED EMBODIMENTS

35 At its most fundamental levels, the invention
comprises genetically engineered antigen preparations of
the zona pellucida and methods of utilizing these antigen
preparations to stimulate the immune system of an animal
and induce the production of antibodies to zona pellucida
antigens in order to prevent fertilization, implantation
or the development of the follicles and subsequent hormone
producing function of the ovaries.

40 The inventor has devised a method of producing
cDNA molecules which code for the zona pellucida antigen
using recombinant DNA techniques. ZP cDNA is inserted
into the expression vectors λ gt 11. This expression
vector is used to transform E. coli. Clones expressing ZP
protein or determinants thereof are identified by ZP
antibody binding. DNA isolated from the single stranded
phage is then used for the expression of the ZP antigen
and as a template for the production of DNA copies and

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1 cDNA. The insert of the λ gt 11 phage DNA encoding the
2 ZP DNA sequence is then inserted into the pEX vector which
3 is used to transform a bacterial host in which the DNA is
4 expressed, producing quantities of the ZP antigen which
5 can be used for immunocontraception.

6 The M13 cloning procedure (Sanger et al., Proc.
7 Acad. Sci. USA 74:5463 (1977)) has been used to determine
8 the partial DNA sequence of three of the 9 cDNA expression
9 clones which have been selected by screening λ gt11
10 expression libraries with affinity purified ZP
11 antibodies. Briefly, this cloning procedure comprises the
12 dideoxy chain termination method in which the zona
13 pellucida DNA is cloned into the filamentous bacteriophage
14 M13.

15 When the zona pellucida genes are expressed in
16 the prokaryotic host E. coli, the polypeptide which is
17 produced is not glycosylated; hence, the molecular weights
18 of the major pig ZP polypeptides are approximately 35, 55
19 and 80 Kd; and the molecular weights of the major rabbit
20 ZP polypeptides are 50, 75, 85 Kd (Table 1) which are
21 lower than that observed for the glycosylated molecules
22 (Table 1). The zona pellucida protein produced in, for
23 instance, prokaryotes from the zona pellucida DNA coding
24 for the zone pellucida polypeptide is termed "rZP" or
25 "recombinant zona pellucida protein." In addition, when a
26 gene coding for the zona pellucida protein is produced in
27 eukaryotes, the protein may be glycosylated and the
28 glycosylated protein is termed "rgZP." The term
29 "immunologically related antigens" is meant to denote
30 those antigens with significant genomic homology to zona
31 pellucida protein such that the products expressed by
32 these DNA show significant levels of immunologic
33 cross-reactivity. An example of such an immunologically
34 related antigen is a polypeptide containing more or less
35 amino acids than the naturally occurring ZP antigen which

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1 has significant immunological or biological cross reactivity with the zona pellucida polypeptide.

The term "host" as used in the present invention is meant to include not only prokaryotes but also 5 eukaryotes such as yeasts and filamentous fungi as well as plant and animal cells.

The term "prokaryote" is meant to include all bacteria which can be transformed with the DNA for the expression of the zona pellucida or rZP protein.

10 The term "eukaryote" is meant to include all yeasts, fungi, animal and plant cells which can be transformed with the DNA for the expression of the zona pellucida, or rZP protein.

15 The DNA for the zona pellucida protein can be derived from any mammalian species. All that is required is that the genetic sequence for the glycoprotein be expressed in the prokaryotic or eukaryotic organism. Preferred is the zona pellucida DNA which expresses ZP protein(s) from pig or rabbit. Especially preferred is 20 the sequence of the zona pellucida DNA which is immunologically cross reactive among multiple animal species (e.g., pig, rabbit, dog, cat or human).

25 A recombinant DNA molecule coding for the zona pellucida protein can be used to transform a host using any of the techniques commonly known to those of ordinary skill in the art. Especially preferred is the use of a vector containing the zona pellucida coding sequence for purposes of prokaryote transformation.

30 The zona pellucida recombinant protein (rZP) of the invention could have more or less amino acids at its flanking ends as compared to the amino acid sequence of native zona pellucida proteins.

35 The term "substantially pure" when applied to the zona pellucida protein of the present invention means that the polypeptide is essentially free of other ovarian

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1 proteins normally associated with the zona pellucida
protein in its natural state and exhibiting constant and
reproducible electrophoretic or chromatographic response,
elution profiles, and antigen activity. The term
5 "substantially pure" is not meant to exclude artificial or
synthetic mixtures of the zona pellucida protein with
other compounds.

Methods for preparing fused, operably linked
genes and expressing them in bacteria are known and are
10 shown, for example, in U.S. Patent No. 4,366,246, herein
incorporated by reference. The genetic constructs and
methods described therein can be utilized for expression
of zona pellucida protein in prokaryotic or eukaryotic
hosts.

15 Prokaryotic hosts may include Gram negative as
well as Gram positive bacteria, such as E. coli, S.
tymphimurium, Serratia marcescens, and Bacillus subtilis.

Eukaryotic hosts may include yeasts such as
Pichia pastoris or mammalian cells.

20 In general, expression vectors containing
promoter sequences which facilitate the efficient
transcription of the inserted DNA fragment are used in
connection with the host. The expression vector typically
contains an origin of replication, promoter(s),
25 terminator(s), as well as specific genes which are capable
of providing phenotypic selection in transformed cells.
The transformed hosts can be fermented and cultured
according to means known in the art to achieve optimal
cell growth.

30 Examples of promoters which can be used in the
invention include, but are not limited to: rec A, trp,
lac, tac, and bacteriophage lambda pR or pL. Examples of
some of the plasmids or bacteriophage which can be used in
the invention are listed in Maniatis *et al.*, Molecular
35 Cloning, Cold Spring Harbor Laboratories, 1982, and others

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1 are known to those of skill in the art and can be easily
ascertained.

5 The invention extends to any host modified
according to the methods described, or modified by any
other methods, commonly known to those of ordinary skill
in the art, such as, for example, by transfer of genetic
material using a lysogenic phage, and which yield a
prokaryote or eukaryote expressing the gene for zona
pellucida protein.

10 A gene is a DNA sequence which encodes through
its template or messenger RNA a sequence of amino acids
characteristic of a specific peptide. The term cDNA
includes genes from which the intervening sequences have
been removed. By the term rDNA is meant a molecule that
15 has been recombined by splicing cDNA or genomic DNA
sequences in vitro.

20 A cloning vehicle is a plasmid or phage DNA or
other DNA sequence which is able to replicate in a host
cell which is characterized by one or a small number of
endonuclease recognition sites at which such DNA sequences
may be cut in a determinable fashion without loss of an
essential biological function of the DNA, and which
25 contains a marker suitable for use in the identification
of transformed cells. Markers, for example, are
tetracycline resistance or ampicillin resistance. The
word "vector" is sometimes used for cloning vehicle.

30 An expression vehicle is a vehicle similar to a
cloning vehicle but which is capable of expressing a given
structural gene in a host, normally under control of
certain control sequences.

35 Hosts transformed with the zona pellucida genome
for zona pellucida proteins are particularly useful for
the production of zona pellucida polypeptide and protein
which can be used for the immunization of an animal. As
stated previously, when the genome for zona pellucida

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1 protein is expressed in bacteria, glycosylation does not occur. Hence the full length recombinant zona pellucida proteins has molecular weights which are lower than the native molecules (see Table 1).

5 The recombinant zona pellucida protein may comprise the entire amino acid sequence of the zona pellucida protein or may comprise only a specific determinant. An animal immunized with zona pellucida recombinant protein will produce antibodies which will 10 bind to epitopes present on the recombinant or naturally occurring polypeptides. Thus, the commercial production of zona pellucida-containing recombinant proteins can be carried out.

15 The term "immunogenically effective amount," as used in the invention, is meant to denote that amount of zona pellucida antigen which is necessary to induce the production in an animal of antibodies which will bind to zona pellucida epitopes.

20 The term "individual" is meant to include any animal, preferably a mammal, and most preferably a cat, dog, cow or human.

25 The zona pellucida recombinant proteins of the invention is particularly useful in sensitizing the immune system of an animal such that, as one result thereof, antibodies reactive with epitopes present on the zona pellucida are produced.

30 Immunization of an individual may be achieved actively or passively. The term "active immunization" means that an antigen or immunogen is administered to an individual and the individual's immune system produces antibodies against the antigen. The immunizing antigen may be any substance to which a body will produce 35 antibodies. Antigens effective in the present invention to actively immunize an individual to produce contraception or sterilization include recombinantly

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1 produced ZP antigen (rZP), whether glycosylated or not, as
well as anti-idiotypic ZP antibodies. The term "passive
immunization" means that antibodies produced outside of
the individual in vitro or in another individual are
5 administered to the individual in order to produce
immunocontraception. The ZP monoclonal antibodies or
genetically engineered single chain antibodies of the
present invention when administered to an individual
produce such passive immunization.

10 When an individual is immunized with ZP antigens
or anti-idiotypic antibodies prior to the maturation of
the ovaries, ovarian development is severely hindered and
permanent irreversible sterilization may occur. This is a
particularly desirable method of causing
15 immunocontraception in animals, particularly pets such as
cats and dogs, when reproduction is undesirable. The use
of ZP immunocontraception in such cases obviates the need
for costly and potentially dangerous surgical
contraception.

20 Preferred for immunocontraception are zona
pellucida peptides which are immunologically cross
reactive among species. Especially preferred are DNA's
encoding the zona pellucida derived from pig or rabbit
ovaries.

25 In another embodiment this invention contemplates
a novel continuous hybridoma cell line which expresses
monoclonal anti-zona pellucida antibody, to the use of
such cell line in production of such antibody, and to a
method for producing such cell line. The invention also
30 contemplates a method for obtaining large amounts of
anti-zona pellucida antibody for use in passive
contraception.

35 According to the present invention a novel
continuous hybridoma cell line which expresses anti-zona
pellucida antibody is obtained by immunizing an animal

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1 with zona pellucida protein, preferably recombinant zona
pellucida protein and most preferably to native zona
pellucida antigens, forming fused hybrid cells between
antibody-producing cells from the immunized animal and
5 myeloma cells, cloning the hybrids and selecting clones
which express anti-zona pellucida antibody. More
specifically, a mouse or other animal is injected with
purified zona pellucida antigen and the antibody producing
cells of the animal's spleen are then fused with a
10 cancerous type of mouse cell or myeloma cell. The hybrid
cell so formed produces the anti-zona pellucida antibody
molecule of its spleen cell parent and continually grows
and divides like its parent myeloma cell. The clone of
cells producing such antibody are selected and grown as a
15 continuous cell line from which large amounts of anti-zona
pellucida antibody is harvested.

20 In the alternative, the clonal hybrid cells may
be injected into a histocompatible animal where they
proliferate, producing high levels of anti-zona pellucida
antibody which can be recovered from the animal's ascites
fluid.

25 Thus, the present invention makes available on a
relatively large scale a reliable and standard supply of
anti-zona pellucida antibody for use in
immunocontraception.

25 The term "immunocontraception" is meant to
include temporary, reversible contraception, and permanent
non-reversible contraception or sterilization resulting
from immunological methods of intervention.

30 This invention includes the use of monoclonal ZP
antibodies for passive immunization resulting in transient
infertility or the use of anti-idiotypic antibodies which
mimic the structure of the native antigen (Erlanger et
al., Immunological Rev. 94: 25, 1986) for active
35 immunization.

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1 The zona pellucida recombinant proteins and
monoclonal antibodies can be administered parenterally by
injection, long release implants, rapid infusion,
intravenously, nasopharyngeal absorption, dermal
5 absorption, and orally. Preparations for parenteral
administration include sterile or aqueous or non-aqueous
solutions, suspensions, and emulsions. Examples of
non-aqueous solvents are propylene glycol, polyethylene
glycol, vegetable oils such as olive oil, and injectable
10 organic esters such as ethyl oleate. Carriers for
occlusive dressings can be used to increase skin
permeability and enhance antigen absorption. Liquid
dosage forms for oral administration may generally
comprise a liposome solution containing the liquid dosage
15 form. Suitable liquid dosage forms include emulsions,
suspensions, solutions, syrups, and elixirs containing
inert diluents commonly used in the art, such as purified
water. Besides the inert diluents, such compositions can
also include adjuvants, wetting agents, emulsifying and
20 suspending agents, and sweetening, flavoring, and
perfuming agents.

It is also possible for the antigenic
preparations containing the zona pellucida recombinant
proteins of the invention to include an adjuvant.
25 Adjuvants are substances that can be used to
nonspecifically augment a specific immune response.
Normally, the adjuvant and the antigen are mixed prior to
presentation to the immune system, or presented
separately, but into the same site of the animal being
30 immunized. Adjuvants can be loosely divided into several
groups based on their composition. These groups include
oil adjuvants (for example, Freund's Complete and
Incomplete), mineral salts (for example, $AlK(SO_4)_2$,
 $AlNa(SO_4)_2$, $AlNH_4(SO_4)$, silica, alum, $Al(OH)_3$, $Ca_3(PO_4)_2$,
35 kaolin, and carbon), polynucleotides (for example, poly IC

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1 and poly AU acids), and certain natural substances (for
example, wax D from Mycobacterium tuberculosis, as well as
substances found in Corynebacterium parvum, Bordetella
pertussis, and members of the genus Brucella).

5 The zona pellucida antigen preparations of the
invention can be used in an animal to induce the
production of antibodies which will bind to epitopic
determinants of zona pellucida. A particularly useful
method in enhancing the production of antibodies to zona
10 pellucida is to first immunize an animal with the zona
pellucida antigenic preparation of the invention followed
by a later second immunization.

The age of the animal at the time of initial
immunization may be critical. For permanent
15 immunocontraception or sterilization, it is most
preferable that the animal be immunized 2-3 months before
the onset of puberty since at this age the most pronounced
interference with ovarian maturation occurs. For
reversible contraception, a ZP antigen expressed late in
20 ovarian follicular development can be used.

One way of determining whether an animal has been
immunized is by determining the animal's immune status
with respect to zona pellucida antigens. This evaluation
can be done by using the zona pellucida recombinant
25 proteins of the invention in an immunoassay such as, for
example, an ELISA assay (Drell and Dunbar, Biol. Reprod.
30:445, 1984) to detect antibodies to zona pellucida. In
so doing, it is possible to determine when the
individual's antibody titer to zona pellucida is
30 sufficiently high to ensure immunization and protect
against pregnancy.

Many different techniques exist for the timing of
the immunizations when a multiple immunization regimen is
utilized. It is possible to use the antigenic preparation
35 of the invention more than once to increase the levels and

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1 diversity of expression of the immunoglobulin repertoire
expressed by the immunized animal. Typically, if multiple
immunizations are given, they will be spaced 1 month to 6
months apart.

5 Generally, the dosage of zona pellucida
recombinant protein administered to an animal will vary
depending on such factors as age, condition, and whether
contraception or ovarian castration is the object of the
immunization, and other variables which can be readily
10 ascertained and adjusted by one of ordinary skill in the
art.

The antigenic preparations of the invention can
be administered as either single or multiple dosages and
can vary from .01-5 g/ml for the zona pellucida antigen
15 per dose, more preferably 0.05-1.0 g/ml zona pellucida
antigen per dose, most preferably 0.1-0.5 g/ml zona
pellucida antigen per dose.

Having now generally described the invention, a
more complete understanding can be obtained by reference
20 to the following specific examples. These examples are
provided for purposes of illustration only and are not
intended to be limiting unless otherwise specified.

Example 1

25 ISOLATION OF ZP PROTEIN AND PREPARATION
OF POLYCLONAL ANTIBODIES

A. Isolation of Zona Pellucida (ZP)

Zona pellucidae (ZP) were isolated using the
30 methods described by Wood *et al.*, Biol. Repr. 25(2):439
(1981).

An instrument consisting of two wheels (10 cm in
diameter) containing 50 rows of ganged razor blades
separated by stainless steel washers (2 mm) was utilized
35 to rupture ovarian follicles. One of the two wheels was

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1 held stationary, while the second was attached to a
rotating handle connected to one of the wheels by a
chain. Ovaries were dropped between the razor blade
wheels submerged in a plexiglass tank containing 6 liters
5 of 0.01 M phosphate buffered saline (PBS), pH 7.2, with 2
mM sodium citrate and 2 mM EGTA. After passing about 300
ovaries through the razor blade wheels, the ruptured
ovaries settled on the bottom of the plexiglass tank on a
removable 1000 μ m mesh nylon screen. The ovaries were
10 then separated from the buffer by removing the screen, and
were washed thoroughly to remove any oocytes which adhered
to the ovaries. The oocytes and zona pellucida were then
isolated by sieving through various sizes of nylon mesh
screens. The oocytes were homogenized and washed through
15 a 50 micron mesh nylon screen so that the zona were
retained. Approximately 100,000 to 300,000 ZP can be
isolated in an 8 hour period using this procedure.

B. Purification of ZP Proteins

20 ZP proteins were purified using high resolution
two dimensional polyacrylamide gel electrophoresis (PAGE)
as previously described by Dunbar *et al.*, Biol. Repr.
24:1111 (1981), incorporated herein by reference. This
method separates proteins by isoelectric focusing (IEF) in
the first dimension and sodium dodecyl sulfate (SDS)
25 polyacrylamide gel electrophoresis (PAGE) in the second
dimension.

30 A pellet of about 30,000 zonae (1 mg protein) was
resuspended in 300 μ l solubilization buffer 2% SDS, 2%
 β -mercaptoethanol, and 1% cyclohexylaminoeth sulfonic
acid (CHES) in water pH about 9.5-9.6). Forty microliters
were applied to each 4% PAGE (with 1.8% bisacrylamide
crosslinker) gel. Ampholines (LKB) with a wide pH range
of 3.5-10 were incorporated into the PAGE gel, and gels
were focused for 16 h at 400 V at 25° C after prefocusing
35 for 2 h at 200 V.

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1 Slab gels (10-20% gradients of polyacrylamide
 with 0.8% bisacrylamide crosslinker) were used for the
 second dimension SDS PAGE. The gels were electrophoresed
 at about 3 amps until the dye front had reached the bottom
 5 of the gel, i.e., at room temperature. Proteins are
 identified by either Coomassie blue staining or silver
 staining. ZP antigens have unique protein profiles which
 exhibit heterogeneity in both charge and molecular weight
 (Table 1) due to their extensive glycosylation. ZP
 10 antigens were identified by immunoblot as shown in Figure
 1 after electrophoretic transfer to nitrocellulose as
 described below. The ZP proteins were eluted from the
 gels electrophoretically. ZP protein N-terminal sequence
 determination was carried out by the method of Hunkapillar
 15 et al., *Meth. Enzymol.* 91:227 (1983).

20 The molecular weights of the major pig ZP
 polypeptides are approximately 35, 55 and 80 Kd; and the
 molecular weights of the major rabbit ZP polypeptides are
 50, 75, 85 Kd (Table 1) which are lower than that observed
 for the glycosylated molecules (Table 1).

TABLE 1

Table of Estimated Molecular Weights of Glycosylated
 and Deglycosylate Pig and Rabbit ZP Proteins

25	Mol. Wt. Range <u>Glycosylated</u>	Enzyme <u>Deglycosylated</u>	TFMS <u>Deglycosylate</u>
<u>Pig</u>			
	ZP I	40-110K	42K
30	ZP II	70-110K	60K
	ZP III	95-118K	80K
<u>Rabbit</u>			
	I	68-125K	65K
	II	81-100K	80K
	III	100-132K	90K

Example 2

Preparation of Polyclonal ZP Antibodies

Polyclonal antibodies were prepared by immunizing 5 female rabbits or castrated male sheep with 300 to 500 µg of total heat solubilized zona pellucida (HSZP) (60-65° C in 0.01M sodium carbonate buffer, pH 9.5 for 1 to 2 hours). Alternatively, approximately 50 to 200 µg 10 of purified ZP proteins isolated by 2D-PAGE gels were used for immunization. All protein samples were suspended in 1 ml water and were emulsified with Complete Freund's 15 adjuvant prior to injection. Animals were injected in multiple intradermal sites and subscapularly and were boosted after 4 weeks with ZP proteins at one half the amount of protein used for the initial immunization in 20 Incomplete Freund's adjuvant. In some instances, additional boosts at monthly intervals using Complete Freund's adjuvant were given to obtain higher antibody titers.

Antibodies were analyzed and characterized using
25 the enzyme-linked immunoassay (ELISA) procedure or
immunoblotting procedure. The ELISA assay used is the
Vecta Stain Kits for detecting rabbit, sheep or mouse
immunoglobulin available from Vector Laboratories. For
this assay 50-100 μ g of HSZP protein in 0.1 M NaCO_3 ,
30 pH 9.0 was added to wells of 96-well microtiter trays.
The assay was then carried out according to methods known
to those of ordinary skill and provided in the kit
instructions. For immunoblotting, the previously
described method of Timmons *et al.*, Biol. Repr. 36:1275
35 (1987), incorporated herein by reference, was used to

1 identify specific ZP glycoproteins and peptides.

Unstained, unfixed 2D-PAGE gels were placed on the cathode side of the nitrocellulose paper (Bio-Rad) and transferred for 2.5 h at 1.3 A using the E-C electroblot transfer unit. The nitrocellulose paper was then blocked overnight in 10 mM Tris(hydroxymethyl)aminomethane (Tris)-saline, pH 7.2, with 3% (w/v) bovine serum albumin (BSA) and 0.01% sodium azide (Tris-saline-azide (BSA)), followed by washing with two changes of Tris-saline-azide (without BSA). Polyclonal serum (1-5 ml), or monoclonal antibody supernatant (containing 100 µg/ml immunoglobulin G (IgG), was diluted in 50 ml Tris-saline-azide (BSA) and incubated with the nitrocellulose transfer at 25° C with shaking overnight, followed by washing with two changes of Tris-saline-azide (without BSA).

15 The nitrocellulose transfer was then incubated with ¹²⁵I-protein A for polyclonal serum, or ¹²⁵I-goat anti-mouse IgG for monoclonal supernatant. A total of 10⁶ cpm in 50 ml Tris-saline-azide (containing BSA) was added to each transfer and incubated overnight with shaking. Extensive washing in Tris-saline-azide (without BSA) was carried out before air drying and exposure to Kodak XAR-5 x-ray film for autoradiography.

20 Antibodies were affinity purified using CNBr activated Sepharose conjugated to ZP proteins.

25 CNBr-activated Sepharose 4B resin (Pharmacia) was used to prepare affinity columns. The resin was coupled to either heat-solubilized HSPZ or HSRZ (heat solubilized rabbit zona pellucida protein). ZP preparations of approximately 1 mg/ml were prepared in solubilization buffer, pH 9.6, as described above. The ZP was suspended in coupling buffer (0.1 M NaHCO₃; 0.5 M NaCl, pH 8.3). The resin was washed and allowed to swell in 1 mM HCl and quickly rinsed with the coupling buffer before application of the ZP in coupling buffer which contained 0.5 NaCl to minimize

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1 protein-protein interaction. The mixture was rotated
slowly at room temperature for two hours. The coupling
buffer with unreacted ZP solution was removed and the
resin was incubated in 1 M ethanolamine overnight at 4° C
5 with slow rotation to block remaining active groups. The
resin was then washed three times each with 1 mM NaHCO₃
(pH 8.3, 0.5 M NaCl), followed by 0.1 M acetate buffer (pH
4, 0.5 M NaCl).

Antiserum was incubated with the coupled resin at
10 4° C under slow rotation overnight. Before elution, the
resin was warmed to room temperature and was washed
thoroughly with borate/saline buffer (100 mM boric acid,
75 mM sodium borate, 75 mM NaCl, pH 8.4) to ensure that
unbound protein was removed. Elution of the bound IgG was
15 carried out using 200 mM glycine (pH 2.7, 0.8% NaCl). The
acid fractions were collected directly into 0.2 M Trizma
base such that the final solution was Trizma:glycine (1:2)
(pH 7.5). This was done to neutralize and thus minimize
damage to the eluted purified antibody.
20 Antibody-containing fractions were aliquoted and frozen
for analysis. Purification of immunoaffinity antibodies
was demonstrated by one-dimensional SDS-PAGE analysis.

Example 3

ISOLATION OF cDNA CLONES EXPRESSING ZP PROTEINS

25 One of skill in the art will recognize that the
method of Chirgwin *et al.*, *Biochem.* 18:5294 (1979) can be
used to prepare RNA from 6 week, 12 week and greater than
6 month old rabbit ovaries. Preferably, ovaries were
30 frozen in liquid nitrogen and pulverized into a fine
powder before homogenization since the ovary contains
significant quantities of connective tissues. From 4 to 8
ovaries were homogenized in 4 M guanidinium thiocyanate
(containing .025 M sodium citrate, 0.5% sodium
35 laurylsarcosine (w/v), pH 7.0) in the presence of

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1 mercaptoethanol. The homogenate was centrifuged at 10,000
rpm in a JA-20 rotor for 10 min at 10° C. The total RNA
was isolated from protein by ethanol precipitation or by
sedimentation through cesium chloride. Optimal yields of
5 RNA were obtained using cesium chloride sedimentation.
The supernatant was layered over 5.7 M CsCl containing 3.8
g/100 mls EDTA (disodium salt) and centrifuged in a SW-40
rotor at 32,000 rpm for 20 hours at 20° C. The pellet is
resuspended in Guanidine hydrochloride and the RNA
10 precipitated with 0.3 ml of 3 M sodium acetate (pH 5.2)
and 2.2 volumes of 95% ethanol. After incubation
overnight at -20° C, the RNA is recovered by
centrifugation at 11500 rpm for 10 min at -5° C. The RNA
is washed 2 times with 95% ethanol, and dissolved in
15 water. Recovery of RNA is monitored by measuring the
260/280 absorbance ratio. Polyadenylated RNA was isolated
by oligo (dT)-cellulose chromatography as previously
described by Maniatis, et al., Molecular Cloning, p. 197
et seq., Cold Spring Harbor Laboratory (1982),
20 incorporated herein by reference. The chromatography
column fractions were monitored at A_{260} with an ISCO
spectrophotometer.

Cell-type specific cDNA probes were prepared as
described by Davis, et al., Proc. Natl. Acad. Sci. USA
25 81:2194 (1984); and Young et al., Proc. Natl. Acad. Sci.
USA 80:1194 (1983). The λgt11 vector used for
preparation of the expression library is shown in Figure
3. For cDNA library construction, 2 micrograms of
30 polyA-mRNA isolated from ovarian tissue was used for each
cDNA synthesis. First strand cDNA was synthesized with
oligo dT priming and addition of reverse transcriptase.
After addition of the reverse transcription and incubation
for 60 min at 43° C, the reaction is terminated by
addition of EDTA. The sample is extracted with
35 phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous

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1 phase is removed, the organic phase reextracted with 0.1 M
1 NaCl containing 10 mM Tris-HCl containing 1mM EDTA (pH
8.0). After pooling the aqueous phases, the DNA is
5 precipitated with 2 M ammonium acetate followed by
addition of 95% ethanol. After freezing and thawing, the
solution is centrifuged, redissolved in 25 μ l of 10 mM
Tris-HCl, 1 mM EDTA (TE). Ten microliters of 7.5 M
10 ammonium acetate and 50 μ l of 95% of ethanol is added.
The RNA-DNA hybrid was then nicked by E. coli RNAase H.
15 Second strand cDNA synthesis was carried out by E. coli
DNA polymerase. The double-strand cDNA was then
blunt-ended by T4 DNA polymerase. The cDNA was methylated
at the EcoRI sites before ligation to EcoRI linkers. The
linkered cDNA was then treated with EcoRI enzyme and
15 purified by chromatography on BioGel P50 (BioRad) and
ligated to λ gt11 arms obtained from Strategene according
to procedures described therewith. Using this procedure
approximately 5×10^3 plaques were obtained for the 6 week
and 8 month old rabbit libraries and 1×10^7 plaques
20 were obtained for the 12 week rabbit library.

20 For library screening, 5×10^3 plaques were
plated per 100mm plate. Plaques were then transferred to
IPTG saturated nitrocellulose paper and probed with
affinity purified antisera prepared as described in
25 Example 2.

25 In order to isolate cDNA's expressing protein
sequences which are similar in multiple species,
polyclonal antibodies against rabbit ZP protein were
affinity purified on porcine ZP columns (ZP protein
30 conjugated to cyanogen bromide activated sepharose
prepared as in Example 2). Antibodies which eluted with
0.1 M glycine (pH 2) are those that recognize antigens
associated with both rabbit and pig ZP proteins.

35 Clones λ gt11-S1, λ gt11-P1, λ gt11-P2 and
 λ gt11-P3 which expressed ZP antigens were subcloned.

1 These subclones were cloned into the M13 phage to sequence
the cDNA using the Sanger dideoxy nucleotide chain
termination method. (Sanger, et al. Proc. Natl. Acad.
Sci. USA 74:5463, 1977). The sequence obtained using this
5 method for three of the cDNA clones expressing ZP antigens
is shown in Figure 4.

Northern blot analysis was used to demonstrate
that the RNA for ZP protein recognized by two of the cDNA
clones (P2 and P3) is present in the ovary but not other
10 tissues. Figure 5 demonstrates one of these analyses
which was carried out using "northern blot" and "dot blot"
hybridization of labeled probe to RNA samples. Briefly,
for the Northern blot analysis, the total RNA was isolated
from ovaries as well as other tissues including liver,
15 kidney, brain, and muscle. The RNAs were denatured and
electrophoresed on a formaldehyde agarose gel and then
transferred from agarose gel to the solid support of
biodyne membrane. The membrane is placed between two
filter papers for the transfer and is then air dried and
20 baked for 2 hours at 80° C. The specific ZP cDNAs which
were inserted into the λgt11 bacteriophage were digested
by EcoRI, and then the insert resolved from the cloning
vector by 1% low melting temperature agarose
electrophoresis. Under UV light, the insert DNA molecules
25 were identified with ethidium bromide and were excised
from the gel for radioactive labeling. cDNA was labeled
according to the random oligo-priming method of Feinberg
and Vogelstein, Anal. Biochem. 132:6 (1983). The labeling
reaction is carried out by addition of denatured cDNA to
30 reagents containing ³²P-dCTP, Klenow fragment, BSA,
mixed primer, and oligo labeling buffer. The labeled DNAs
were separated from unincorporated dCTP by chromatography
on a column of Sephadex G-50. For DNA-RNA hybridization,
the RNA transferred biodyne membranes are placed into
heat-sealable polyethylene bags separately. The
35

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1 prehybridization solution is added into the bag and the
bag immersed in a 42° C. water bath overnight. The
prehybridization solution was removed and replaced with
hybridization solution containing ^{32}P -labeled DNA probe
5 and immerse in 42° C. water bath overnight. Finally, the
membrane was washed several times and exposed to X-ray
films with lightning plus screens. The film was exposed
for 48 hours at 70° C prior to development.

10 Example 4

Expression of ZP Proteins by Recombinant DNA

Recombinant ZP proteins (rZP) are expressed in
these prokaryotic or eukaryotic expression systems.

Recombinant λ gt11 phage, λ gt11-S1 λ gt11-P1,
15 λ gt11-P2 and λ gt11-P3 prepared as described in Example
3 are used to infect E. coli Y1089 by incubating the phage
with E. coli Y1089 for 20 min at 32° C. in Luria Broth
(LB) medium containing 10mM MgCl_2 . Colonies are grown
at 32° C and single colonies tested for temperature
20 sensitivity by transfer to 42° C. Colonies that grow at
32° C but not at 42° C are considered to be lysogenic, and
comprise approximately 10-70% of the population. Single
colonies of the recombinant lysogen are isolated, used to
inoculate LB medium, and grown at 32° C. The culture is
25 transferred to 42° C when the O.D. 600 is about 0.5 and
incubated a further 20 min. Transcription of the cloned
gene is stimulated by the addition of isopropyl
 β -D thiogalactopyranoside (IPTG) to a final
concentration of 10 mM and the culture incubated at 37° C.
30 for 60 min. The transformed E. coli are harvested by
centrifugation, resuspended in 50 mM Tris (pH 7.5) and
frozen. Lysis occurs upon thawing of the cells, releasing
the fusion ZP protein.

The bacteriophages containing ZP DNA designated
35 λ gt11-P1 and λ gt11-P3 were deposited with the American

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1 Type Culture Collection (ATCC), Rockville, Md., U.S.A.,
Deposit Accession Nos. 40377 and 40378, respectively, on
October 8, 1987. The deposits are available pursuant to
the patent laws and regulations of the United States and
5 of those countries foreign to the United States in which
counterparts of this application are filed. The
availability of a deposit does not constitute a license to
practice the invention of this application in derogation
of any patent issued thereon or on any division or
10 continuation of this application.

In another embodiment the pEX plasmids described
by Stanley and Luzio, EMBO 3(6):1429 (1984) is used to
transform E. coli Y1090. The pEX vector expresses a
hybrid protein which is insoluble. Isolation of
15 bacteriophage λ DNA will be carried out on the
recombinant λ gt11 phage following the procedure
described by Maniatis *et al.*, Molecular Cloning: a
Laboratory Manual, Cold Spring Harbor Laboratory (1982),
incorporated herein by reference. E. coli Y1090 grown in
20 LB medium supplemented with 10mM MgCl₂, 0.2% maltose and
50 μ g/ml Ampicillin (Amp) are infected with recombinant
 λ gt11 phage and incubated at 37° C for 20 min. Infected
E. coli are plated on LB plates in LB media with 0.7%
agarose, MgCl₂ and Amp, and incubated overnight at 43°
25 C. Phage are collected in SM media (0.02M Tris-HCl, pH
7.5; 0.01M NaCl; 0.001M MgSO₄.7H₂O) (pH 7.5) and 0.01%
gelatin in 1 liter water) and bacteria removed by
centrifugation at 8,000 \times g for 10 min at 4° C. RNase A
30 and DNase I are added to the supernatant containing the
phage at final concentrations of 1 μ g/ml and incubated
for 30 min at 37° C. An equal volume of SM containing 20%
polyethylene glycol and 2 M NaCl is then added and the
suspension incubated at 0° C for 1 hr, followed by
centrifugation at 10,000 \times g for 20 min at 4° C. The
35 supernatant is removed, and pellet containing the phage

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1 resuspended in 0.5 ml M medium. To remove remaining
debris, the solution is centrifuged at 8,000 x g for 2 min
at 4° C, and 5 μ l of 10% SDS and 5 μ l 0.5 M EDTA (pH
8) is added to the supernatant and incubated at 68° C for
5 15 min. Sequential extractions with phenol,
phenol:chloroform:isoamyl alcohol (25:24:1), and
chloroform:isoamyl alcohol (24:1) are performed,
harvesting the aqueous phase in each step. An equal
volume of isopropanol is added to the aqueous phase and
10 the solution stored at -70° C for 20 min. After
centrifugation for 10 min at 4° C the pellet is washed
with 70% EtOH, dried, and resuspended in 50 μ l TE.
EcoR1 restriction enzyme digestion is carried out
following standard procedures on an aliquot of the
15 isolated DNA. After digestion is complete, Tris boronate
EDTA loading buffer (TBE) 90 mM Tris borate, 90 mM boric
acid, 2 mM EDTA) is added at 1:1 ratio and loaded on 1.4%
agarose gel. The gel is run at 100V until the xylene
cyanole front is midway through the gel, the DNA bands are
20 visualized by ultraviolet light and bands of the predicted
size removed. Electroelution will be carried out in the
Elutrap apparatus (S&S) in TBE. The DNA fragments
isolated are then inserted into pEX plasmids.

Plasmid preparation is carried out following the
25 procedure in Maniatis (1982), incorporated herein by
reference. After growing a bacterial clone overnight, 1.5
ml of the culture is placed in an Eppendorf tube,
centrifuged for 1 min, and the supernatant removed by
aspiration. The pellet is resuspended in 100 μ l of an
30 ice-cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris
(ph 8.0), and 4 mg/ml lysozyme, and incubated at 22° C.
for 20 min, followed by addition of 200 μ l of ice-cold
0.2 M NaOH and 1% SDS. Following incubation on ice for 5
min., 150 μ l of 3M potassium acetate (pH 4.8) is added.
35 The solution is incubated on ice for 5 min, followed by

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1 centrifugation and transfer of the supernatant to a fresh
tube. 600 μ l phenol:chloroform:isamylalcohol (25:24:1)
is added and the solution mixed and centrifuged, saving
the supernatant. Two volumes of cold ethanol is then
5 added to the supernatant, and the solution mixed and
incubated at 22° C for 2 min. The supernatant is removed
and the pellet dried by inverting the tube. The pellet is
washed in 70% EtOH, mixed, centrifuged, and the pellet
again drained. 50 μ l of TE (pH 8.0) containing 20
10 μ g/ml DNase-free pancreatic RNase is added, mixed
briefly and an aliquot removed for digestion by EcoRI.
Analysis of the digestion is carried out by agarose gel
electrophoresis.

The plasmid is linearized with EcoRI (ProMega
15 BioTech) under conditions specified by the manufacturer.
200 ng of linearized plasmid DNA is added to a 3-fold
molar excess of cDNA isolated as described above. The DNA
is precipitated with EtOH and resuspended in 8 μ l of TE
(pH 8.0). One μ l of 10x ligation 66 mM Tris-Cl (pH
20 7.6), 6.6 mM MgCl₂, 10 mM dithiotheitol, 66 mM ATP)
buffer is added, followed by addition of 10 units of T4
DNA ligase. The mixture is incubated at 12° C for 8
hours. A one μ l aliquot is analyzed by agarose gel for
ligation. 2.4 μ l are used to transform bacteria by the
25 CaCl₂ technique.

Colonies carrying the recombinant plasmid are
identified by screening bacteria with available polyclonal
antisera. Transformed cells are grown for 20 hours at 30°
C. Nitrocellulose is overlayed and the filter removed and
30 placed colony side up under 2 layers of 3 MM filter paper
presoaked in LB media containing 100 μ g Amp/ml.
Transcription of the hybrid protein is started by
incubation at 42° C. for 2 hours. The filter will be
washed 3x for 30 min each in TBS and blocked in TBS
35 supplemented with 2% dry milk overnight. The filter is

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1 then probed with affinity purified antibody. Amplification
of positive colonies is carried out by incubation at 32°
C. Expression of ZP proteins is induced by transfer to
42° C. for 2 hours, at which time, the recombinant ZP
5 protein accounts for about 25% of the total SDS extracted
protein. The expressed hybrid ZP protein is insoluble and
is then easily purified by conventional chromatographic
methods.

Alternatively, recombinant ZP DNA can be inserted
10 into yeasts. The methylatrophic yeast such as Pichia
pastoris can be used for efficient and large scale
expression of genetically engineered proteins. M.
Bluestone and P. Savage, Chemical Week, McGraw-Hill, Inc.
(1986). These yeast utilize methanol as a base brew which
15 the yeast metabolizes into formaldehyde with the help of
an alcohol oxidase enzyme. A second enzyme will then
convert the formaldehyde into dihydroxyacetone, for the
next stage of yeast cell synthesis. The cDNA can be
inserted in place of the gene that makes the alcohol
20 oxidase so that recombinant DNA from the ZP protein can be
substituted at the site where the alcohol oxidase gene
would normally be. Because this site would be modified in
yeast would still respond to methanol only therefore
switching to ethanol or to a carbohydrate food source
25 would cause expression or non-expression of the inserted
gene. The expression of the lacZ gene from two
methanol-regulated promoters can now be carried out in
Pichia pastoris as described by Tschopp et al., Nucleic
Acids Research 15(9):3859 (1987).

30

Example 5

PURIFICATION OF RECOMBINANT ZONA PELLUCIDA PROTEIN

Recombinant ZP protein is expressed as described
in Example 4.

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1 The λ gt 11 recombinant lysogen was used to
infect E. coli Y1089. The E. coli was grown in LB medium
supplemented with 0.2% maltose at 32° C to an optical
density (600 nm) of 0.5. The culture was then shifted to
5 42° C and incubated for an additional 20 minutes. At the
end of this time IPTG was added to derepress the lac
repressor so that the lacZ-directed gene containing the
recombinant is expressed. The cells were incubated at
37-38° for 60 minutes and cells were frozen in liquid
10 nitrogen and thawed to lyse the cells. The lysate was
collected in 0.1 M Tris buffer (ph 7.5) and passed over a
Sephacel column and the void volume containing protein
having molecular weights greater than 100 Kd was
collected. Since the expressed protein will be a fused to
15 β -galactosidase protein in this case, this is a rapid
way to partially purify this protein. This protein
fraction is then analyzed by SDS-PAGE and immunoblotting
to detect the expressed antigen. Figure 6 shows a
SDS-PAGE immunablot of a ZP antigen-fusion protein
20 expressed by the P1 clone. The nitrocellulose transfer
was probed with rabbit anti-HSPZ. Multiple low molecular
weight bacterial proteins are recognized in all bacterial
proteins are recognized in all control samples, but the
two lanes with the P1 insert show expression of a ZP
25 specific fusion protein.

26 Protein will be isolated from the pEX plasmids by
extracting the protein from the bacteria. Because the
recombinant cro- β -galactosidase hybrid protein accounts
for about 25% of sodium dodecyl sulfate extracted protein,
30 it can be precipitated and run over Sephadex and ion
exchange columns or with preparative electrophoresis to
purify the protein sufficiently to purify for immunization
of animals. The recombinant protein isolated from the
yeast culture media could also be purified by these
35 methods.

Example 6

IMMUNIZATION WITH *Zona pellucida*

Active immunization with zona pellucida protein has been shown to be effective in inhibiting fertilization in animals. Active immunization of rabbits also results in the impairment of ovarian follicular development and therefore steroid hormone production in rabbits. (Figure 7).

ZP protein (300 µg/0.5 ml; 0.1 M PBS) is emulsified in 0.5 ml Complete Freund's adjuvant (CFA).
10 Half of the dose is administered intradermally in multiple sites, and half was injected subscapularly. Control animals are injected with an emulsion consisting of 0.5 ml CFA emulsified with 0.5 ml PBS. The animals receive a
15 boost immunization 4 weeks after the primary injection. This boost included a preparation identical to the initial one except that Freund's Incomplete Adjuvant is used. In order to evaluate ovarian function, animals are treated with hCG, and serum progesterone levels were determined.
20 Some animals of each group were killed at least 12 hours after hCG administration, and ovaries were weighed and examined for ovulation sites. Alternatively, animals received porcine FSH (Reheis Chemical Co., Phoenix, AZ; 0.5 mg/animal twice daily for 3 days) before hCG to induce
25 superovulation. The results of these studies show that ovarian function is altered as evidenced by lack of follicle cell differentiation and steroid production.
30 (See Figure 7.). Serum progesterone levels were determined in rabbits immunized with porcine ZP. Responses of animals given 50 IU hCG were determined by RIA of progesterone levels. Serum samples tested were taken periodically during the 21-day period in which pseudopregnancy normally follows such hCG treatment. Each histogram represents the mean \pm S.D. of serum progesterone concentration (nanograms of progesterone per ml serum).
35

-36-

1 Panels A and D show progesterone profiles of control
2 (adjuvant only) and experimented (ZP-immunized) animals,
3 respectively, before immunization. B and E show similar
4 determination made 20 weeks after primary immunization. C
5 and F show progesterone profiles of nonboosted animals
6 only after FSH/hCG treatment 28 weeks after primary
7 immunization. The use of the monoclonal antibodies R5 and
8 R7 for passive immunization can also result in reduced
9 fertility levels in rabbits. The monoclonal antibody
10 designated PSI is made against a silver stained pig ZP
protein purified by 2D-PAGE.

This monoclonal antibody inhibits the attachment
of pig sperm to pig ZP in vitro (Figure 8).

15

Example 7

PREPARATION OF ANTI-ZONA PELLUCIDA MONOCLONAL ANTIBODIES

16 The procedure for preparing a hybrid cell line
17 which produces anti-zona pellucida monoclonal antibodies
18 involves fusion of myeloma cells of a BALB/c mouse with
19 the spleen cells of BALB/c mice primed with zona pellucida
20 protein. The procedure is also applicable using myeloma
21 cells and anti-zona pellucida antibody producing cells
22 from other sources such as humans, dogs or cats.

A. PREPARATION OF SPLEEN CELLS FOR FUSION

23 The zona pellucida proteins was purified as the
24 heat solubilized total matrix from ovaries or from
25 separation by high resolution 2D-PAGE. The zona pellucida
26 antigen was about 95% pure (heat solubilized ZP) or 100%
27 pure (2D-PAGE analysis) based on 2D-PAGE and silver stain
28 analysis. The zona pellucida antigen was used to immunize
29 adult BALB/c or C57B46 male mice by subcutaneous
30 administration of about 30 ug emulsified in Freund's
31 complete adjuvant. The mouse was reimmunized 2 weeks
32 later with a further 30 ug of the protein in incomplete
33 adjuvant given subcutaneously. After an additional 2-6
34 weeks the mouse was killed and the spleen removed.

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1 weeks, 20-40 ug of the protein were administered
intravenously, and 2-4 days later the mice were sacrificed
and a spleen cell suspension was prepared in the manner
taught by Gefter, et al., Somatic Cell Genetics 3:231,
5 1977. Red blood cells were lysed for incubation of 15
minutes at 40° C. in NH₄Cl (0.83%). The resulting cell
suspension was washed by centrifugation (800 x g) through
heat-inactivated calf serum followed by centrifugation in
protein-free medium (RRMI 1640, buffered with 7.5 mM
10 HEPES, pH 7.2).

10 B. PREPARATION OF MYELOMA CELLS FOR FUSION
Myeloma cells derived from the P3U1 line and
deficient in HPRT (E.C.2.4.2.8) as described by Yelton, et
al., Curr. Top. Microbiol. Immunol. 81:1-7 (1978), were
15 maintained in Eagle's minimum essential medium (MEM)
containing 10% fetal calf and 15% horse serum. The growth
of myeloma cells is inhibited by selective
hypoxanthine-aminopeterin-thymidine (HAT) medium.

16 C. PRODUCTION OF HYBRIDS
Production of hybrids was accomplished by mixing
20 10⁷ BALB/c myeloma cells with 10⁸ spleen cells
obtained from the zona pellucida immunized BALB/c or C57
Bl/6 mice. The cell mixture was centrifuged at 800 x g
and the cells were resuspended for fusion in a 50%
25 solution (w/v) of polyethylene glycol (PEG 1000) diluted
in minimum essential medium (MEM) without serum following
the procedure described by Gefter et al. (1977). The
resulting hybridoma cells were cloned in
hypoxanthine-aminopeterin-thymidine (HAT) medium by
30 limiting dilution as described by Galfre and Milstein
Meth. Enzymol. 73:3, 1975. Two hybridoma cell lines R5
and PS1 have been selected because the cell line R5
produces an antibody which recognizes the protein portion
of ZP antigens of multiple animal species. The antibody
35 PS1 appears to recognize a carbohydrate determinant on

-38-

1 multiple species of ZP and this antibody also inhibits
sperm from binding to the surface of the ZP. (Figure 8).

5 The hybridoma cell lines designated R5 and PSI
were deposited with the American Type Culture Collection
9566, and HB 9565, respectively, on October 8, 1987. The
deposits are available pursuant to the patent laws and
regulations of the United States and of those countries
foreign to the United States in which counterparts of this
10 application are filed. The availability of a deposit does
not constitute a license to practice the invention of this
application in derogation of any patent issued thereon or
on any division or continuation of this application.

15 D. TESTING OF THE CLONES FOR PRODUCTION OF ANTI-ZONA
PELLUCIDA ANTIBODY

20 Linbro (Flow Lab) microtiter 96 well plates were
coated with 50-100 ug of total rabbit or pig ZP protein
and incubated at 20° C. overnight. After washing the
wells three times with 0.1 M Tris (pH 7.5) - 1% nonidet
P-40, containing 5% Carnation Instant Milk and 0.085
25 sodium azide, 0.05 ml of the culture supernatants were
added and incubated at 40 C. overnight. The supernatant
was removed after washing three times with RIA buffer and
the antibodies were detected using the Hybridoma Screening
Kit (Bethesda Research Labs). Controls for non-specific
25 binding were included by omitting either the second
antibody or the culture supernatant.

30 The hybridoma cells designated R2, R5, R7 and PSI
were grown as an ascites form by intraperitoneal injection
into pristane-treated mice (Galfre and Milstein, Meth.
Enzymol. 73 (part B) 1 (1981)), and the resulting ascites
fluid was used as a source of the monoclonal antibody for
immunoblots and biological assays.

35 The invention now being fully described, it will
be apparent to one of ordinary skill in the art that many

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1 changes and modifications can be made thereto without
departing from the spirit or scope of the invention as set
forth below.

5 WHAT IS CLAIMED AS NEW AND IS DESIRED TO BE
COVERED UNDER LETTERS PATENT IS:

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-40-

1 1. A recombinant polypeptide comprising the
a amino acid sequence of the zona pellucida antigen.

5 2. A recombinant polypeptide comprising the
a amino acid sequence of the zona pellucida protein
substantially free of native glycosylation.

10 3. The polypeptide of any of claims 1 or 2 in
substantially pure form.

15 4. The polypeptide of claims 1 or 2, wherein
said zona pellucida protein is encoded by DNA of ovaries
of a mammal.

15 5. The polypeptide of any of claims 1 or 2,
wherein said amino acid sequence is encoded by the nucleic
acid sequence shown in Figure 4.

20 6. An expression vehicle which comprises a DNA
sequence coding for the zona pellucida antigen.

25 7. The expression vehicle of claim 6, wherein
said expression vehicle is a phage or plasmid capable of
replication in a host which comprises, in operable
linkage:
a) an origin of replication;
b) a promoter; and
c) a DNA sequence coding for the zona pellucida
antigen.

30 8. The expression vehicle of claim 7, wherein
said expression vehicle is a phage or plasmid capable of
replication in a prokaryotic host which comprises, in
operable linkage:

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- 1 a) a prokaryotic origin of replication;
- b) a prokaryotic promoter; and
- c) a DNA sequence coding for the zona pellucida antigen.

5 9. The expression vehicle of claim 8, wherein said expression vehicle is selected from the group consisting of λ gt11-S1, λ gt11-P1, λ gt11-P2 and λ gt11-P3.

10 10. A vector comprising a DNA sequence coding for a zona pellucida protein.

11. The vector of claim 10 wherein said vector is isolated from the group consisting of a plasmid, a 15 phage and a cosmid.

12. The vector of claim 10, wherein said vector is λ gt11-P1 having Accession number ATCC 40377.

20 13. The vector of claim 10, wherein said vector is λ gt11-P3 having Accession number ATCC 40378.

14. A host transformed with a recombinant DNA 25 molecule wherein said recombinant DNA molecule comprises a DNA sequence coding for the zona pellucida antigen.

15. The host of claim 14, which is a prokaryote.

30 16. The host of claim 15, which is an eukaryote.

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1 17. The host of any of claims 14 or 15, wherein
said zona pellucida antigen is encoded by the nucleic acid
sequence shown in Figure 4.

5 18. The host as in any of claims 14-17, wherein
said recombinant DNA molecule is a phage.

10 19. The host of claim 14, which is E. coli.
20. The host of claim 14, which is a yeast.

21. A method of producing zona pellucida protein
which comprises:
15 a) transforming a host with a DNA sequence
coding for zona pellucida protein;
b) expressing said DNA sequence; and
c) recovering said zona pellucida protein.

22. The method of claim 21, wherein said host is
20 a prokaryote.

23. The method of claim 21, wherein said host is
a eukaryote.

25 24. A pharmaceutical composition useful for
inducing the production in an individual of antibodies to
zona pellucida antigen comprising an immunogenically
effective amount of zona pellucida antigen produced by the
method of claim 21.

30 25. The pharmaceutical composition of claim 24
wherein said antigen is recombinant zona pellucida
protein.

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1 26. The pharmaceutical composition as in any of
claims 24 or 25, wherein said pharmacological carrier
contains an adjuvant.

5 27. A method of inducing the production of
antibodies in an individual to zona pellucida comprising
immunizing said individual with the pharmaceutical
composition as in any of claims 24, 25 or 26.

10 28. A continuous cell line which produces a
monoclonal antibody which specifically binds to zona
pellucida antigen comprising a fused cell hybrid of spleen
cells from an individual immunized with zona pellucida
antigen, and myeloma cells.

15 29. The continuous cell line of claim 28 which
produces said monoclonal antibody in vivo by injection of
said cells of said cell line into a histocompatible animal
from which said antibody is capable of being recovered
20 from the ascites fluid of said animal.

25 30. An anti-zona pellucida monoclonal antibody,
which binds to zona pellucida, produced by a continuous
cell line comprising a fused hybrid of rodent spleen cells
immunized with zona pellucida antigen, and rodent myeloma
cells.

30 31. An anti-zona pellucida monoclonal antibody,
which binds to zona pellucida, produced by a cell line
having the identifying characteristics of ATCC HB 9565.

35 32. The method of producing contraception in an
individual comprising administration to said individual a
contraceptive dose of anti-zona pellucida monoclonal
antibody.

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1 33. The method of producing contraception in an
individual comprising administration to said individual a
contraceptive dose of the polypeptide of any of claims 1
or 2.

5

 34. The method of claim 33 wherein said
individual is selected from the group consisting of a cow,
pig, cat and dog.

10 35. The method of claim 33 wherein said
individual is a human.

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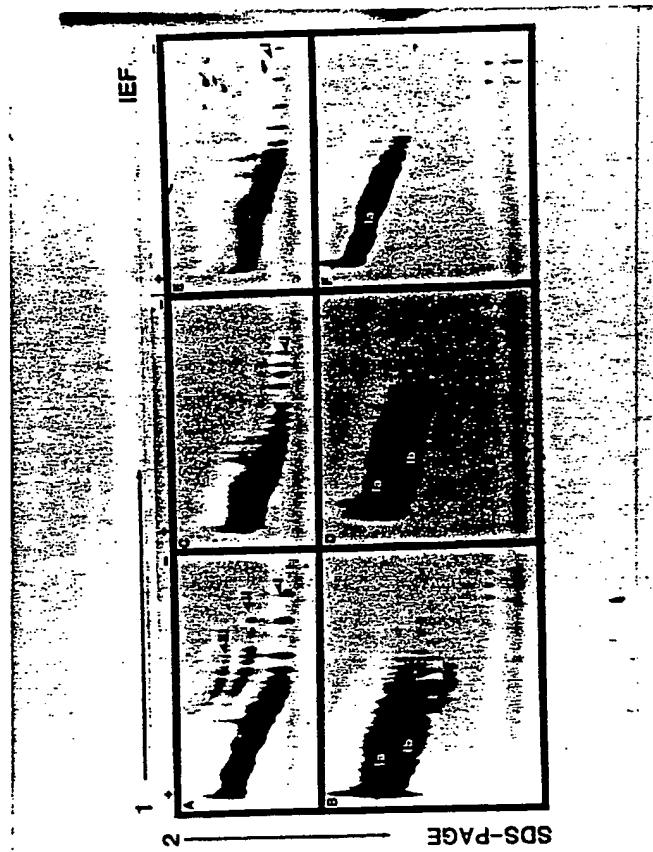


FIG. 1

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N-TERMINAL AMINO ACID SEQUENCES OF RABBIT ZP GLYCOPROTEINS

I (E) Lys-Gln-Pro-Lys-Pro-Glu-Thr-Por-Thr-Asp-Pro-Gly-Val-Leu-His-X-Arg-Pro-Pro-Asn-Phe-Lys-Phe-Thr-Ile-

II (E) Lys-Gln-Leu-Gln-Pro-Ser-Asp-Pro-Ala-Phe-Pro-Gly-Thr-Val-His-X-Asn-Glu-

III (E) N-blocked

E : Deglycosylated with endo beta-galactosidase
X : Amino acid residue undetermined

FIG. 2A

N-TERMINAL AMINO ACID SEQUENCES OF PIG ZP GLYCOPROTEINS

I (E) X-Val-Pro-Thr-Ile-Gly-Leu-Cys-Asp-Ala-Val-Pro-Val-Trp*-Asp-Arg-Leu-Pro-Cys-Ala-Pro-
Leu ArgII (T) X-Val-Pro-Thr-Ile-Gly-Leu-Cys-Asp-Ala-Val-Pro-Val-Ser*-Asp-Arg-Leu-Pro-Gln-Ala-Pro-Pro-Pro*-Asp-
Leu Arg

III (E) X-X-Asn-Val-Lys*-Arg-Glu-Asp-Ser*-X-Gln-Arg-Met*-Gly-Gly-Ser-

III (E) X-X-Pro-Gln-Leu-Val-Asn-Thr-Ala-Phe-Pro-X-Ile*-Val*-

E : Deglycosylated with endo beta-galactosidase

T : Deglycosylated with trifluoromethane sulfonic acid

X : Amino acid residue undetermined

* : Amino acid residue identification tentative

FIG. 2B

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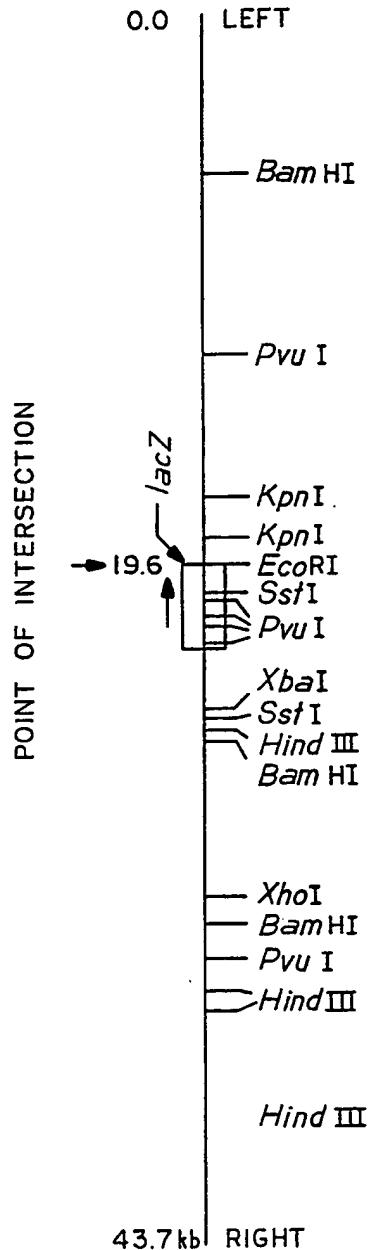


FIG. 3

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S1

(313 BASES)

1 TAGGAGACCA CATCTTTTA GATACACTTC CTGCTTGCCT TCTGTTAGAG GTAGCTACTT
 61 CACCTACAGG GTCTCCTGC AAATTTATTT CTTCCACAGA TTTCTGAGGA CTTGTATGAC
 121 TGAGGCAAGT GGAGGATGCT AGTGAATGAA TGCTATTGTT TTTCAATATT GATGAAGCAA
 181 TGCACTCCATC ACTTTTTAAT TTCAATTAGTA GGTTCTCTCA ATTTTGTCTC CTGATTCTA
 241 CATTCTGAGT TCACAAGATC AAGGATCATC TGTACACAAG TACCGTGTAT GTTAGTGATG
 301 TGTCACACAC AGA

(352 BASES)

1 ATGGGACAAA GCCCAGAAGA GAGGGGTGAA GCTTGTTCCT GTTCTCTGGG TGGAGAAATG
 61 CAGGACAGCT GGAGCACACA TTGGATGAGT CATTATTCCC TGCAAGCAAT ACCAGTGAGC
 121 ACTTACCAAG TCTGATTAAG AAAAGCGCA AGTGTATGCA GCCCAAAGAT TTTTTCCCA
 181 AAACACCAGA TAATGATAAA AGACTTCAAA AGAAATTGAA CAAAATGGCT CAAGAACTAC
 241 AGAGGCAAAA AACCACTCTA GATAATGATA CGCCTGTTCT CTTATTGAT CTAATGATAT
 301 GTTGACGTTA TAGTCCCACA GTTAAGTGTG TAGTCACACAC GCTCATGGAG GA

P2

(830 BASES)

1 AGAAATGGACA GTGCACTGCT ATTACACCAAG AGATGACATG CTACTCAATG CCAATATCAA
 61 AAGTCTTCCT CCTCCTGTGG CCTCGTGAAG CCAGGTCAC TTGCTTGTG CCTGCAAACC
 121 TACCCAGATG AGTCTCTATCA ACAGCCTTAC AGGGTCAATG AGTACCCAT AGTGAATATAC
 181 CTCCGCCAAC CAATTACAT GGGAAAGTGAAG AGTCCTAAAT AGAAATGACC CCAATATCAA
 241 GCTGGCCCTA GATGACTGCT GGGCTAACAT CTTCATGGAC CAGCATCTCT CCCCAGTGG
 301 AGTATTGTCA TGGACCGCTG TGACTATAGC CTGGACACTA TCAACTAAT TCACCAAGTTG
 361 GCTCTCTGTG ACCTATCCCT AGCACTTAC GAGGTTGTGAT GTGAAGACTT TGCTTTGTAT
 421 CAGAGGCCCA AGCACGCTCT AGCCTGGGCT AACTTCCACT GCAGTGCCTT AATCTGCAAT
 481 CAACATGCAG CTGGCAATCA ACAACTATCC TGACTCTCT TGCTCTGTGA CTTGCCCTGG
 541 TCATCTAGAC ACAGCAGGCC ACAGGAACAC CGAAGAGGAG AGAGTGCACAG CCAGCCTCCC
 601 AGGACCCATT CACCTCACCT GTTACCGAAT GGCTCTTCCT TCAGAGGTGT TGGGGATTGG
 661 AAGGAGCATG CCATGGCTGG GATGTTACTT CTAAAACAAT GGCTGCTGTG GCTGCCGTAG
 721 CAGGTGTAGT GGCAACTCTA GGCTTCATCA GTTACCTGTG TAAAAAGAGG ACCATGATGT
 781 TAAGTCACTA ACTTGACTTG CAAATAAAAT GGTTGAAATA AAAAAAAAAA

P3

(272 BASES)

1 TTTAAGTTTA CTATAAATTT TCAGAACCCAG GAGACAGGGT ATTCCCTGT ACTGTAACCT
 61 GGGACAAACCA AGGGCGATTG CACACGCTGC AGAGATGACA CTGACTGTGG CACCCGGTA
 121 GGAGAGGGTC CAGGCCCTC CTGTTGACTG GAAGCAAACAT ACAGCAGCTG CTACGTCACT
 181 GAGTCGGAAC CGTACTACGT CATGCTGGTC GGGGTTGAAG AGGTGGACGC AGCTGGACAA
 241 AACCTGGTTA CAAAGCAGCA GCTGCTTAAG GT

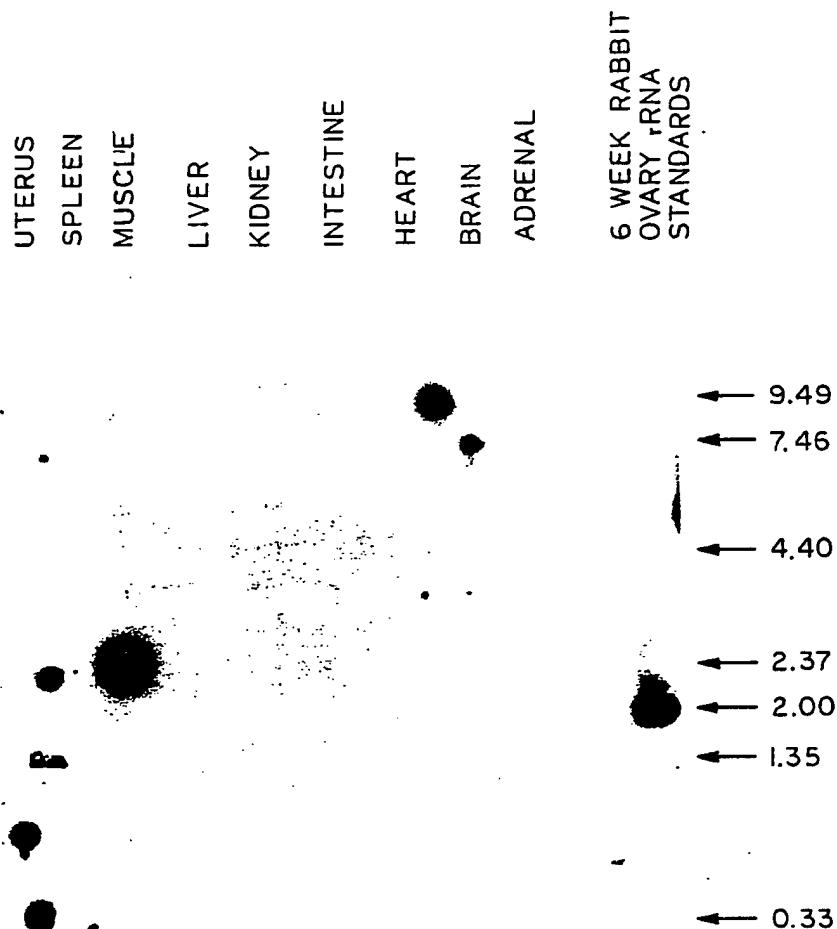
(484 BASES)

1 CCGATGCCCTA CACTGCGACA CTTACAACCA GCTATCCAGT CAGAGCTCGA TCTGCATCCC
 61 TCTCACACAG CGCTCAGCAT TCACCTCAGC TTCTGATCCT CATGCAAGA GGCTCTAAAG
 121 GACCGTATAT CTGCACTGCA GTGTGTCACT CTGCCAGCCT ACTGGGACAC AATCCTGTAC
 181 GGTAACCTGT CCTATTGACA GTCGAAGAAG AAACCTGGAC ACATCAATT CCAGAACAGT
 241 ACTGCTAACAA TTTCTAGCAA GGACCCATGA TTCTACTTCA AGGCCACAGA GGATCCCTCA
 301 GAAAGCTGGA TAAACACGCA GTGTTCTCT GCATCTGAG CTCTATGGGT GGCAGGCCCT
 361 TCTGGGATCT TCATTGGAGC CTTGTATCCT ATGTGTGGCA ATCAGGACAC GAAGATGAGT
 421 TCCCTGGGCC AATATCATC AATATATAAC TCAGATGTGC ACCACCAAA AAAAAAAAAA
 481 AAAA

FIG. 4

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NORTHERN BLOT. mRNA OF DIFFERENT TISSUES
PROBED WITH LABELED CLONE P3

FIG. 5

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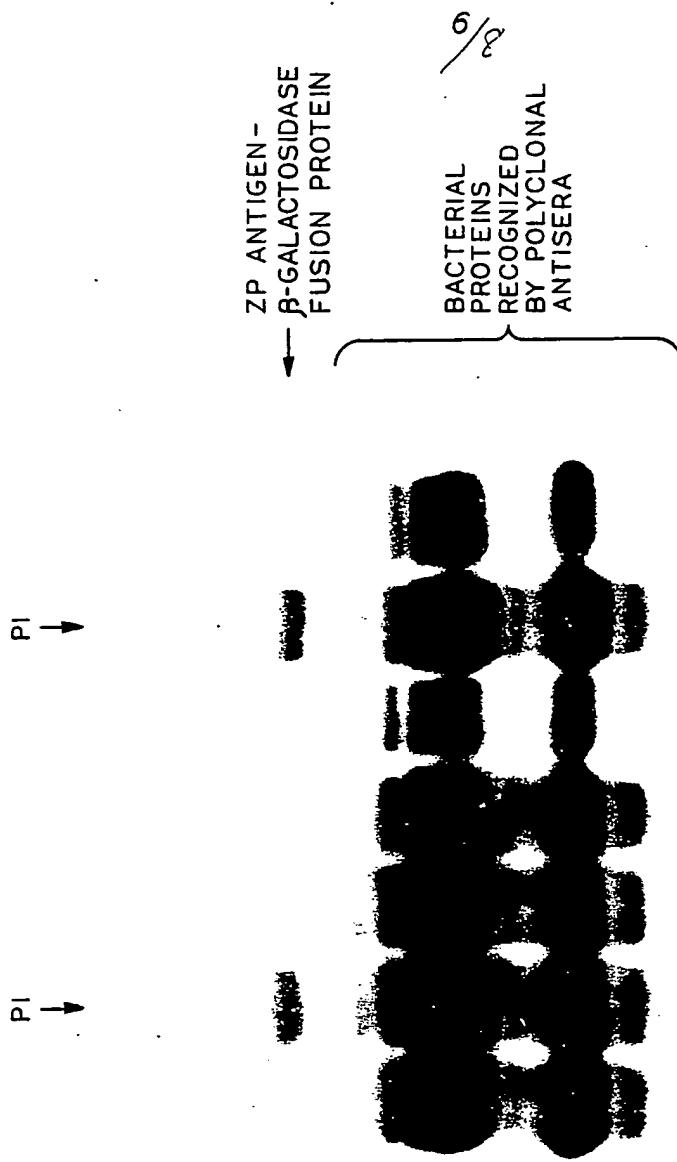


FIG. 6

SDS-PAGE IMMUNOBLOT OF PROTEIN EXPRESSED FROM
λgt11 DNA FROM CONE PI PROBED WITH SHEEP ANTISERA
TO HEAT SOLUBILIZED ZONA PELLUCIDA

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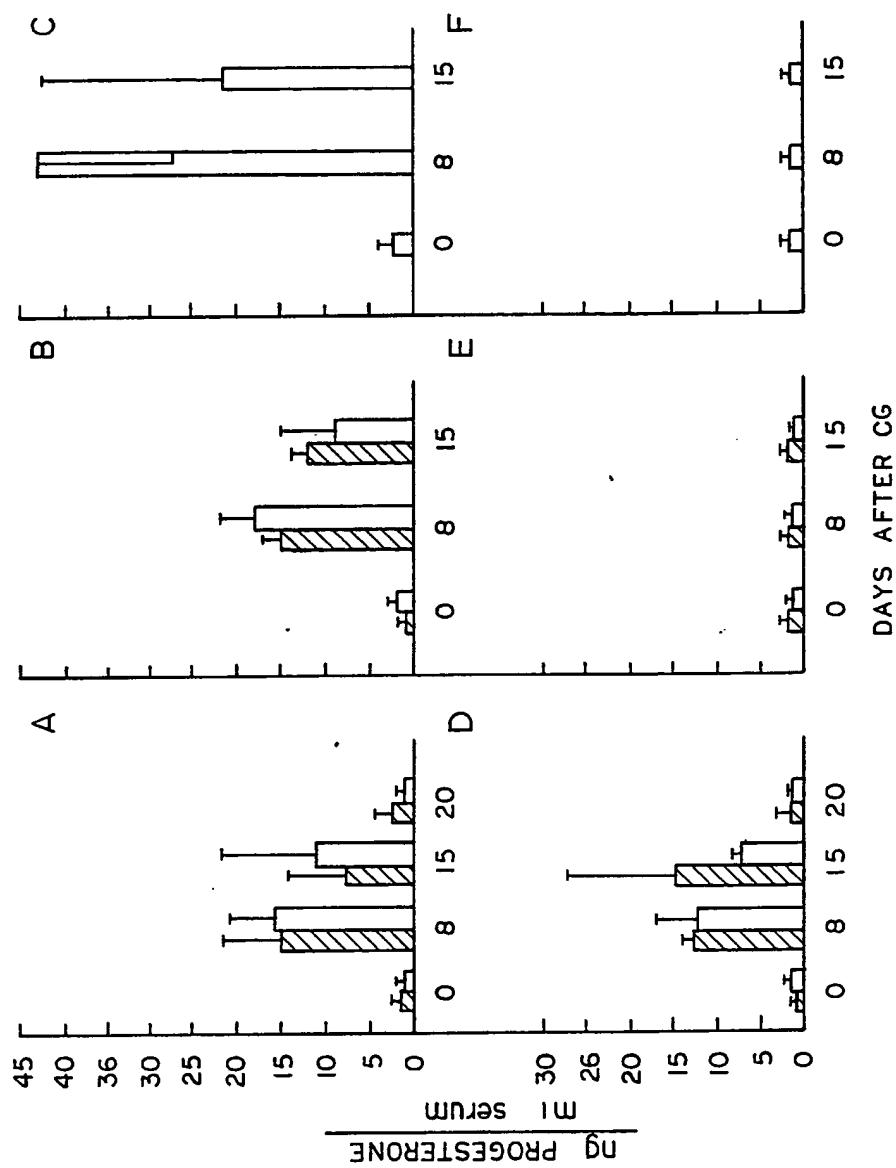


FIG. 7

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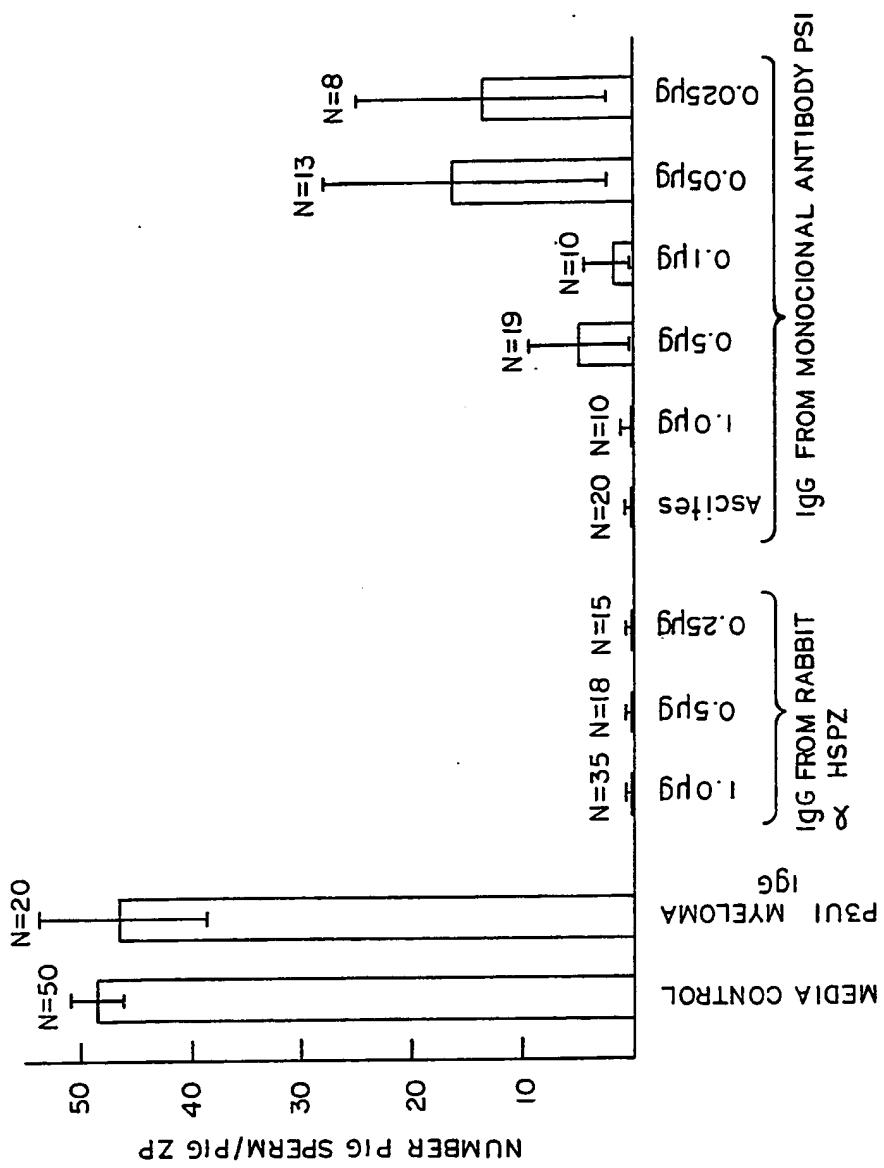


FIG. 8.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/03285

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶	
According to International Patent Classification (IPC) or to both National Classification and IPC ⁶ A61K 39/00; C07K 13/00 . IPC (4): C07K 15/14; C12P21/00; C12N 15/00, 7/00, 5/00, 5/02; C07H15/12; U.S. CL: 424/85, 88; 435/68, 172.3, 240.27, 320; 530/397, 328, 387	

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
U.S.	435/68, 172.3, 240.27, 320; 530/397, 328, 387; 424/88, 85
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸	

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	US, A, 3,992,520 (MERCK AND CO., INC.) 16 November 1976 (see Abstract, Introduction, and claims).	24-26, 33-35
A	US, A, 4,248,864 (AMERICAN HOME PRODUCTS CORP.) 3 February 1981 (see Abstract, Introduction, and claims)	24-26, 33-35
X	WO 87/05516 (APHTON CORP) 24 September 1987 (see Abstract, Introduction, and claims)	1-5, 24-35
X	Proc. Natl. Acad. Sci. (USA), Volume 83, No. 12, issued 20 June 1986 (Washington, D.C.), (RINGUETTE ET AL), "Oocyte-specific gene expression: Molecular characterization of a cDNA coding for ZP-3. The sperm receptor of the mouse Zona pellucida: See Abstract, Introduction, and Figure 1.	1-23

- Special categories of cited documents: ¹⁰
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 December 1988	16 FEB 1989
International Searching Authority	Signature of Authorized Officer
ISA/US	Gary R. Fabian

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Chemical Abstracts, Volume 100, No. 17, issued 23 April 1984 (Columbus, Ohio, USA) (EAST ET AL) "Monoclonal antibodies as probes for the distribution of ZP-2. The major surface glycoprotein of the murine zona pellucida", see page 414, column 1, the abstract No. 136355 K, J. Cell. Biol. 1984, 98 (3), 795-800 (Eng.).	30-32
Y		24-26
		28-29
X	Chemical Abstracts, Volume 106, No. 9, issued 2 March 1987 (Columbus Ohio, USA), (VUREWICZ ET AL) "Structural characterization of the Mr=55,000 antigen (ZP-3) of porcine oocyte -zona pellucida, "see page 253, column 2, The abstract No. 63282w, J. Biol. Chem. 1987, 262(2), 564-571 (Eng.).	1-5
Y		33-35
X	Chemical Abstracts, Volume 103, No. 11, issued 16 September 1985 (Columbus, Ohio, USA), (KOYAMA ET AL) "Production and characterization of monoclonal antibodies to cross-reactive antigens of human and porcine zona pellucidae", see page 473, column 1, the abstract No. 86212a, J. Reprod. Immunol. 1985, 7 (3), 187-198 (Eng.).	30-32
Y		28-29
		1-5

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter^{1,2} not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out^{1,3}, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See attachment.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.

Attachment to PCT/ISA/210

Part VI. Observations Where Unity of Invention is Lacking

Group I, claims 1-23, drawn to a peptide product, a vector and transformed host used in a method of making the peptide, classified 435/172.3,68.

Group II, claims 24-26, drawn to a pharmaceutical composition, classified 424/88.

Group III, claim 27, drawn to a method of inducing the production of antibodies, classified 424/88.

Group IV, claims 28, 29, drawn to a cell line, classified 435/240+.

Group V, claims 30,31, drawn to a monoclonal antibody, classified 530/380+.

Group VI, claims 32-35, drawn to a method of contraception, classified 424/85.

\$700.00 payment approved by Rosanne Goodman on 23 November 1988 for additional Groups II-VI; charge to Deposit Account No. 06-2375 . Counsel advised that she has no right to protest for any group not paid for and that any protest must be filed no later than 15 days from the date of mailing of the search report.

Reasons for holding lack of unity of invention:

The inventions listed as Groups I to VI do not meet the requirements for Unity of Invention for the following reasons: the claims are drawn to multiple products (a peptide, a DNA sequence, a monoclonal antibody, a pharmaceutical composition) and multiple uses of the products (making the peptide, generating antibodies, use as a contraceptive). Applicant is only allowed one product, a method of making and a method of using said product. Note PCT Rule 13.1 and 13.2

Time Limit For Filing A Protest

Applicant is hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2 applicant may protest the holding of lack of unity only with respect to the groups(s) paid for.